Network-Level Changes in Expression of Inducible Fos–Jun Proteins in the Striatum during Chronic Cocaine Treatment and Withdrawal

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Summary

Repeated exposures to psychomotor stimulants produce long-term changes in behavior ranging from addiction to behavioral sensitization. Many of these behaviors depend on the nigrostriatal system of the basal ganglia. We show here that chronic cocaine exposure not only leads to time-varying alterations in the inducibility of bZIP transcription factors in individual striatal neurons, but also to long-lasting network changes in which ensembles of striatal neurons express these proteins. These network-level adaptations suggest that the behavioral sensitization induced by repeated psychomotor stimulant exposure may reflect an enduring functional reorganization of basal ganglia circuits.

Introduction

Increasing evidence suggests that mesostriatal dopamine systems are involved in behavioral learning as well as in the expression of learned behavioral routines. The mesolimbic system, which projects from the ventral tegmental area to the nucleus accumbens, has been implicated in reinforcement-based behaviors and addiction (Self and Nestler, 1995; Wise, 1996). The nigrostriatal system is also involved in reward-based sensory-motor conditioning and motor responsiveness to addictive drugs (Robinson and Becker, 1986; Kalivas and Stewart, 1991; Graybiel, 1995). Work in the monkey has further suggested that sensory-motor conditioning may involve changes in the relative responsiveness of neurons in the two major neurochemical compartments of the striatum, the striosomes and the matrix (Aosaki et al., 1995).

To approach the mechanisms underlying such plasticity, research in rodents has focused on studying the molecular changes that occur when animals are treated chronically with psychomotor stimulants. These drugs increase extracellular dopamine and other monoamines in the striatum by blocking the dopamine transporter (the main effect of cocaine) and by increasing dopamine/monoamine release (a main effect of amphetamine) (see Giros et al., 1996). Behaviorally, chronic exposure to psychomotor stimulants produces augmented motor responses to further exposure to the stimulants, a phenomenon called behavioral sensitization (Robinson and Becker, 1986; Kalivas and Stewart, 1991; Cador et al., 1995). This change in behavior can last for months or longer. At the cellular level, chronic treatment with these stimulants produces increased activity in the D1-class dopamine receptor transduction pathway involving activation of the cyclic AMP (cAMP)-dependent protein kinase (PKA) cascade with subsequent activation of the cAMP response element–binding protein (CREB) through phosphorylation at Ser-133 (Cole et al., 1995; Self and Nestler, 1995). Other receptors have been implicated as well, including dopamine D2-class receptors (Ruskin and Marshall, 1994), serotonin (Bhat and Baraban, 1993), and glutamate NMDA receptors (Torres and Rivier, 1993; Wang et al., 1994). Changes in level of expression of several immediate-early genes have also been noted (Hope et al., 1994; Rosen et al., 1994).

Many of the molecules so far associated with the striatal plasticity induced by psychomotor stimulants have also been implicated in neuroplasticity and long-term memory in other systems across a range of vertebrates and invertebrates (Bear and Malenka, 1994; Frank and Greenberg, 1994; Bartsch et al., 1995; Huang and Kandel, 1995; Yin et al., 1995). However, it is still not clear how these common mechanisms are selectively engaged by different neural circuits to bring about appropriate adaptive responses.

The striking changes in behavior associated with behavioral sensitization suggested that the phenomenon might reflect a change in the circuit-level activity of the basal ganglia. In the experiments described here, we asked whether one could obtain anatomical correlates of such a circuit-level change by tracking changes in the expression of immediate-early genes in the striatum of rats developing behavioral sensitization. Our findings demonstrate that during chronic treatment with cocaine and during drug withdrawal, Fos–Jun family proteins undergo coordinate network-level changes in expression that occur with the known time course of behavioral sensitization. These findings suggest that the neuronal plasticity underlying behavioral sensitization may involve functional reorganization of basal ganglia circuits.

Results

Striatal Expression of Immunodetectable c-Fos, JunB, and FRAs Stimulated by Acute Cocaine Treatment

To obtain baseline data for the experiments on the effects of chronic cocaine exposure, we treated rats acutely with cocaine (25 mg/kg) and after 2 or 18 hr processed their brains for immunocytochemistry. Following a single dose of cocaine, there was a rapid increase in neuronal expression of c-Fos, JunB, and Fos-related antigen (FRA) immunoreactivities in the caudoputamen above control levels (Figures 1A and 1B). The acute induction patterns for c-Fos, JunB, and FRA differed in their intensities and kinetics (Figure 1A). The intensities followed the order c-Fos < JunB < FRA. The c-Fos and JunB immunoreactivities rapidly disappeared, but immunoreactivity detected with the FRA M

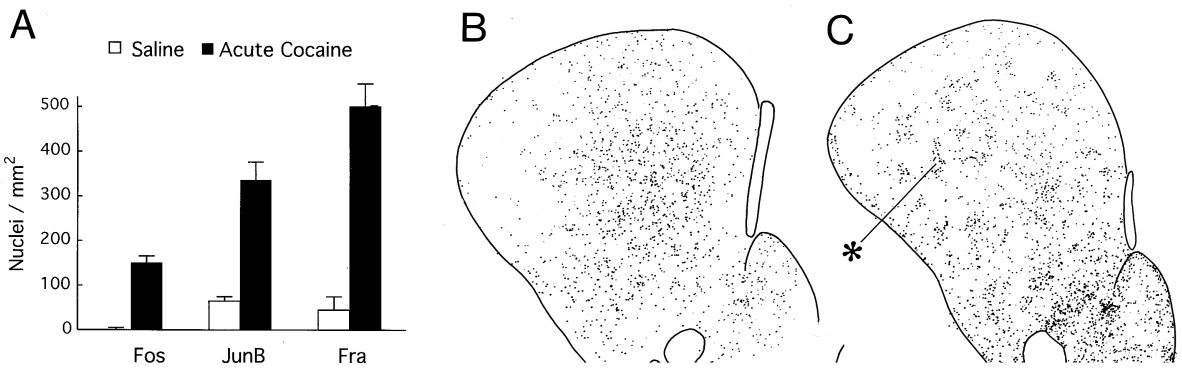

Figure 1. Acute Exposure to Psychomotor Stimulants Induces Different Quantitative Levels of Fos–Jun Protein Expression in the Striatum, in Distribution Patterns Specific to the Psychomotor Stimulant

(A) Numbers of nuclei immunopositive for c-Fos (Fos), JunB, or FRA (Fra) counted in sections from the brains of rats treated with a single dose of cocaine (25 mg/kg, closed bars) or saline (open bars), 2 hr before perfusion (n = 4–5 per group). Values shown are means ± SEMs. (B and C) Distributions of JunB-positive nuclei induced in the rat caudoputamen by acute cocaine (25 mg/kg) (B) and acute amphetamine (5 mg/kg) (C). Note the widespread distribution of JunB-positive nuclei induced in the central caudoputamen after acute cocaine (B), as compared to the clustered striosomal distribution after acute amphetamine treatment (C). Asterisk in (C) indicates one such striosomal cluster. Charts in (B) and (C) and in subsequent figures are prints from high contrast photographic negatives.

peptide antiserum was present at levels above baseline for at least 18 hr (see Figures 4D and 5A). The long-lasting FRA immunoreactivity was nuclear and confined to neurons, as were the c-Fos-, JunB-, and FRA-like immunoreactivities detected 2 hr after stimulation.

We used the regional patterns of distribution of the induced proteins as an assay to determine which striatal neurons were activated by the cocaine treatment (Figure 1B). We tested for the selectivity of these cocaine-evoked patterns by comparing them with those found in rats given acute amphetamine (Figure 1C), which induces a different pattern of expression. We found that the increases in expression for all three protein immunoreactivities occurred in the drug-specific patterns characterized previously for c-*fos* and *NGFI-A* mRNAs (Graybiel et al., 1990; Moratalla et al., 1992, 1993). Acute treatment with cocaine induced extensive, relatively homogeneous expression, with strong immunolabeling in the central and middle parts of the caudoputamen and weak labeling laterally (Figure 1B). By contrast, acute treatment with amphetamine (5 mg/kg) led to expression in a striosome-predominant pattern in the rostral and lateral caudoputamen (Figure 1C). This documentation of similar drug-specific patterns of induction for the three protein immunoreactivities proved to be critical for the analysis of their patterns of expression following chronic treatment with cocaine.

Inducibility of Immunodetectable c-Fos, JunB, and FRAs in the Caudoputamen during and after Chronic Cocaine Treatment

To study the dynamic regulation of c-Fos, JunB, and FRAs by chronic exposure to cocaine, we gave repeated 25 mg/kg doses twice daily for 7 days, and on the next day (18 hr later) gave a single dose of cocaine followed by a 2 hr survival time. To test for early changes in the inducibility of the three protein immunoreactivities, we challenged other rats on day 5.

The numbers of c-Fos-positive nuclei found 2 hr after cocaine challenge on day 5 were markedly reduced relative to the numbers of immunoreactive striatal neurons detected following a single acute dose of cocaine (Figure 2). The numbers of JunB-positive and FRA-positive nuclei were not appreciably changed. By day 8, the inducibility of c-Fos-like immunoreactivity was almost completely lost (~92% decrease) and the number of JunB-positive nuclei was reduced by over half (~53%), but the number of FRA-positive nuclei was nearly unchanged (~8% lower). Thus, the latency and degree of response of the three protein classes, as measured by immunostained nuclei expressing them, differed sharply during chronic treatment.

Changes in the Compartmental Patterns of Expression of Fos–Jun Family Proteins in the Caudoputamen Following Chronic Exposure to Cocaine

Despite the distinctly different changes in inducibility of c-Fos-, JunB-, and FRA-like proteins that occurred with chronic cocaine treatment, all three classes of protein underwent coordinate changes in their anatomical expression patterns in the striatum. The most striking finding was a shift in distribution of immunostained cells from the broad distribution characteristic of typical acute cocaine cases (Figures 2B and 2C) to a more patchy distribution (Figures 2B′ and 2C′).

We compared these altered distributions to the locations of the striosome and matrix compartments of the striatum by analysis with calbindin D_{28K} as a marker for the matrix compartment and dynorphin as a marker for striosomes. These comparisons (Figures 3A–3B′) showed that the changes in expression pattern with chronic cocaine treatment involved a decrease of induction in the matrix relative to nearby striosomes and an increase of induction in striosomes.

The altered pattern of expression following chronic cocaine treatment and challenge was remarkably similar to the pattern found after acute amphetamine treatment

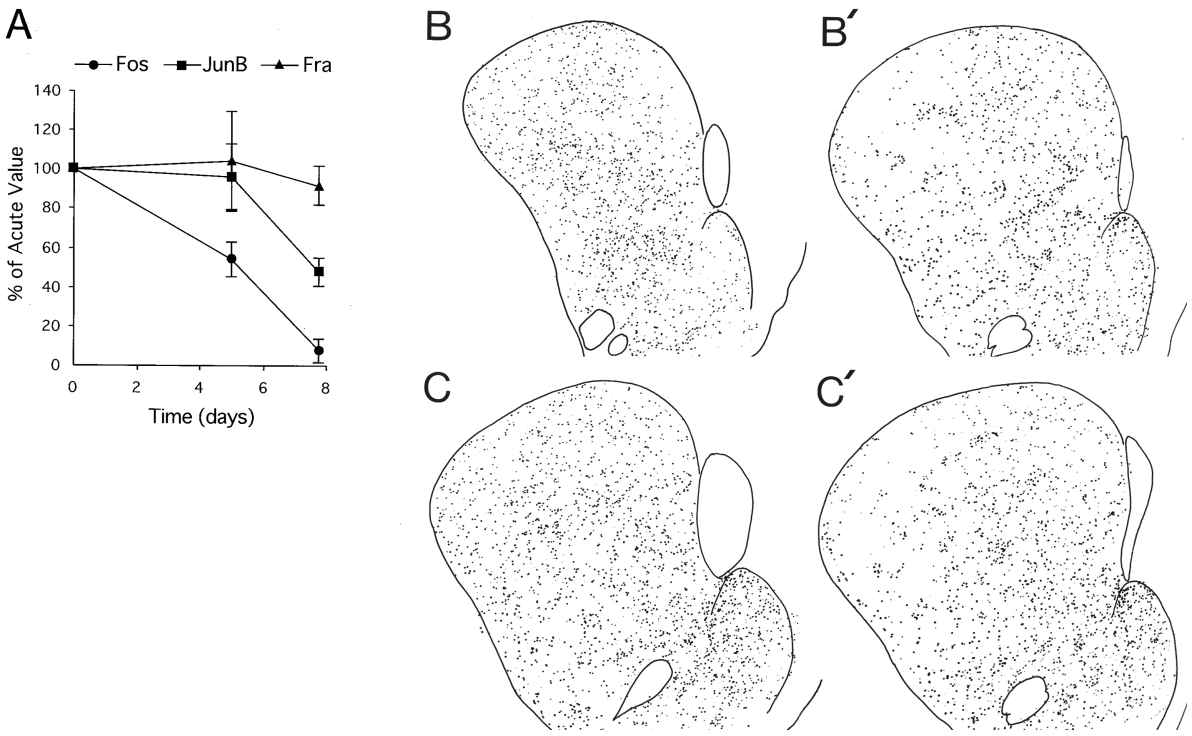

Figure 2. Changes in the Distribution and Levels of Expression of Fos–Jun Proteins Occur during Chronic Treatment with Cocaine

(A) Numbers of nuclei immunopositive for c-Fos (Fos), JunB, and FRA (Fra) during (day 5) and after (day 8) chronic cocaine treatment compared to the numbers induced by acute cocaine (day 0). Values indicate means ± SEMs (n = 4–6) and are shown as percents of the corresponding immunopositive nuclei found in acutely treated rats.

(B–C′) Charts illustrating the distribution of JunB-positive nuclei. (B and B′) and FRA-positive nuclei (C and C′). (B) and (C) show sections from rats given acute cocaine (25 mg/kg) following 7 days of treatment with saline. (B′) and (C′) illustrate sections from rats given the same dose of cocaine following 7 days of treatment with cocaine. Note change from relatively homogeneous pattern of induction in acutely treated rats (B and C) to patchy patterns of expression of JunB and FRA with chronic cocaine treatment (B' and C'). All rats were perfused 2 hr after the last dose of cocaine.

(cf. Figure 1C with Figures 2B′ and 2C′). The increased relative expression in striosomes was strongest in the anterior and lateral parts of the caudoputamen, and augmented patchiness was never pronounced medially. The pattern shift was already visible on challenge on day 5 in the sections immunostained for JunB and for FRAs (data not shown). For the severely down-regulated c-Fos-like immunoreactivity, the patchy pattern was also evident on challenge on day 5, but only scattered c-Fos-positive nuclei remained by the end of the chronic treatment (data not shown).

These results demonstrate that after repeated exposure to cocaine, a single further dose of the same drug activates different populations of neurons in the striatum than activated by cocaine in drug-naive controls. Thus, the pattern of induction of Fos–Jun proteins by cocaine that has been repeatedly demonstrated for acute cocaine treatment is not a fixed property of the striatal response to the drug, but changes with repeated drug exposure.

Long-Term Effects of Chronic Cocaine Treatment on the Inducibility and Distributions of Fos–Jun Family Proteins in the Caudoputamen

The observed redistribution of c-Fos-, JunB-, and FRA-immunoreactive neurons, the nearly complete down-regulation of c-Fos-like immunoreactivity, and the persistent expression of FRA-like proteins during chronic cocaine treatment and withdrawal may be indicative of striatal neuroplasticity occurring during the course of behavioral sensitization. To test this hypothesis, we treated a group of rats chronically with cocaine for 7 days, allowed withdrawal periods of 18 hr or 3, 7, or 14 days, and then gave a final challenge with cocaine 2 hr before sacrifice. Control rats were chronically treated with saline and given cocaine challenge at the same timepoints. Qualitatively, the rats treated chronically with cocaine, but not the control rats, showed augmented behavioral responsiveness to the drug challenge during withdrawal.

In the caudoputamen, immunoreactive c-Fos, which had become refractory to induction by cocaine 18 hr after chronic treatment, responded to the challenge dose at roughly 60% of the acute level of response by 3 days of withdrawal and reached only ~70% of acute levels of inducibility after 2 weeks (Figure 4A). The numbers of JunB-positive nuclei present after cocaine challenge, depressed by about 50% at the end of treatment, showed steady recovery during withdrawal and reached values slightly above those of the acutely treated controls by 2 weeks (Figure 4A). The numbers of FRA-positive nuclei, the least reduced by the chronic treatment,

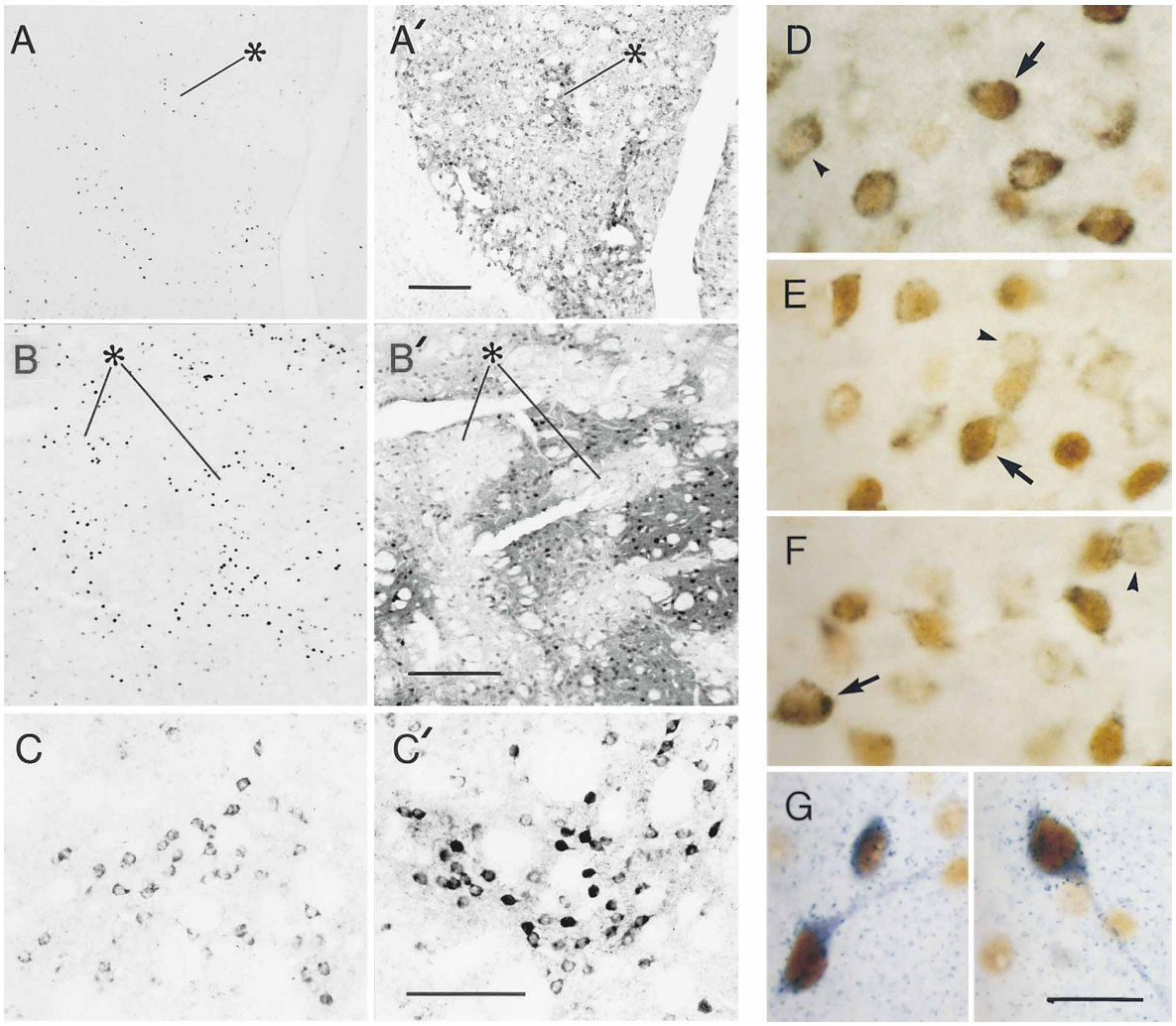

Figure 3. Chronic Cocaine Treatment Results in Long-Lasting Redistribution of Inducible Fos–Jun Protein Expression and Augmented Dynorphin Expression in Striosomes

(A–B′) Clusters of FRA-positive (A) and JunB-positive (B) nuclei in lateral caudoputamen of rats chronically treated with cocaine, matching dynorphin-positive (A′) and calbindin D_{28K}-negative (B′) striosomes in serially adjacent sections. Rats were given cocaine (25 mg/kg, b.i.d.) for 7 days and perfused 18 hr after the last cocaine injection (A and A′) or were then withdrawn from cocaine for 1 week and challenged with a dose of cocaine (25 mg/kg) and perfused 2 hr later (B, B′, and D–G). In (A)–(B′), asterisks indicate examples of corresponding clusters.

(C and C′) Dynorphin-positive cell clusters in the caudoputamen of a rat treated with saline for 2 weeks (C) and a rat treated with cocaine (25 mg/kg, b.i.d.) for 2 weeks and perfused 2 hr after the last injection (C′). Note the greater intensity of the dynorphin immunostaining in neurons of the chronically treated rat. Scale bars indicate 200 μm in (A′) and (B′) and 100 μm in (C′).

(D)–(F) document coordinate expression of c-Fos (D), JunB (E), and FRA (F) immunoreactivities (brown nuclei) in dynorphin-positive projection neurons (black). Examples of doubly labeled neurons are shown at arrows. Examples of dynorphin-immunoreactive neurons without such nuclear immunostaining are shown at small arrowheads. (G) illustrates three NADPH diaphorase-stained neurons (blue) with FRA-positive nuclei (brown). Scale bar for (D)–(G) (in [G]) indicates 20 μm.

fell slightly until after 1 week of withdrawal, and then rose rapidly to slightly above control values at 2 weeks (Figure 4A).

Strikingly, the distribution of immunoreactive neurons induced by cocaine challenge during the withdrawal period continued to exhibit the pattern of augmented striosomal expression and decreased matrix expression that had developed during chronic treatment (Figures 3B, 3B′, 4B′, and 4C′). This patchy pattern was evident at the earliest timepoints during withdrawal for JunB- and FRA-positive nuclei (data not shown). The new pattern became evident even for the severely down-regulated c-Fos as soon as appreciable numbers of c-Fos-immunopositive nuclei became detectable on subsequent challenge during withdrawal (3 days). For all three classes of protein, the change in pattern of expression, once initiated by the chronic treatment, persisted throughout the entire 2 week withdrawal period (Figures 4B–4C′). Thus, even when the counts of immunopositive neurons had recovered to levels near or above those found on acute treatment (as found for JunB and FRA), or had at least undergone partial recovery (as found for c-Fos), the distributions of the immunopositive neurons remained changed. The appearance of the pattern

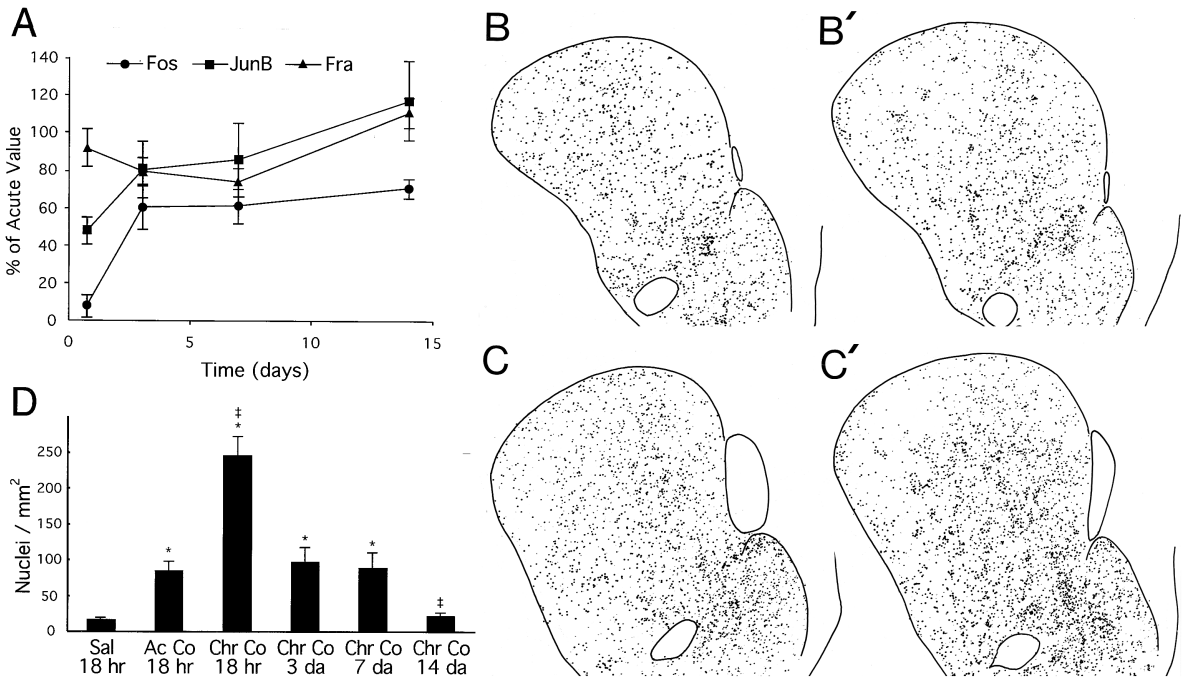

Figure 4. Inducibility of Fos–Jun Proteins and Persistence of FRA Proteins in Striatal Neurons Undergo Time-Varying Changes during Withdrawal from Chronic Cocaine Treatment

(A) Numbers of c-Fos-, JunB-, and FRA-immunoreactive neurons in the caudoputamen of rats given a cocaine challenge (25 mg/kg) 18 hr or 3, 7, or 14 days after withdrawal from chronic cocaine treatment (25 mg/kg, b.i.d., for 7 days). Values are plotted as a percent of those in acutely treated rats (n = 4–6 per group).

(B–C′) Charts comparing the distributions of JunB-positive nuclei (B and B′) and FRA-positive nuclei (C and C′) in the caudoputamen of rats given acute cocaine (B and C) or a challenge of cocaine (25 mg/kg) given after 2 weeks of withdrawal (B′ and C′). Note that the patchy distribution of the immunoreactive nuclei in the cocaine-treated animals persists through the full 2 week period of withdrawal.

(D) Quantitative data showing that persistent FRA-like immunoreactivity is detectable by immunohistochemical analysis for up to 1 week of withdrawal. Bars indicate numbers of immunopositive nuclei/mm^2 counted in samples from different treatment groups (n = 4–6 per group). Rats were injected with cocaine (25 mg/kg, b.i.d.) for 7 days and were perfused without challenge 18 hr or 3, 7, or 14 days after the last cocaine injection. Control rats received saline injections for 7 days and were perfused 18 hr later. An asterisk indicates values different from saline-treated control values at $p < 0.05$; a double dagger indicates values different from acute cocaine 18 hr treatment group values at $p < 0.01$. Statistics by two-tailed t tests.

change immediately after the chronic treatment (JunB and FRA) and its continuation throughout the withdrawal (Fos, JunB, and FRA) suggest a time course paralleling that of behavioral sensitization rather than that of augmented intrastriatal dopamine levels, which have been shown not to appear until roughly day 3 of withdrawal in a similar experimental protocol (Kalivas and Duffy, 1993).

Persistence of FRA but Not c-Fos or JunB Immunoreactivities in Striatal Neurons Following Chronic Cocaine Treatment

Studies with the FRA antiserum we used have shown that chronic cocaine treatment induces FRA proteins with long half-lives (Hope et al., 1994; Zhang et al., 1992; Rosen et al., 1994; Nye et al., 1995). To test for such a build-up, we treated rats with cocaine for 7 days and analyzed the immunoreactivities detectable 18 hr later or after 3, 7, and 14 days of withdrawal with no further challenge. There was no increase in the numbers of c-Fos-positive or JunB-positive neurons above background counts at the 18 hr timepoint or later (data not shown). By contrast, there were large numbers of FRA-positive neurons 18 hr after the end of the 7 day chronic treatment (Figure 4D). By day 3 of withdrawal, the numbers of FRA-positive neurons were reduced by about 50%. They remained practically unaltered at day 7 and then returned to control levels by day 14.

There was clearly augmented striosomal expression of the persistent FRAs, especially in lateral striosomes at the 18 hr survival times, when enough FRA-positive nuclei were still present to score the pattern (Figures 3A and 3A′). This result suggests that both persistent FRAs and transient FRAs were subject to compartment-selective regulation as a result of the cocaine treatment.

Phenotypes of Neurons Expressing c-Fos, JunB, and FRA Immunoreactivities Following Chronic Exposure to Psychomotor Stimulants

Acute treatments with cocaine and amphetamine induce c-Fos-like immunoreactivity in a restricted subset of neurons in the caudoputamen. These are the dynorphin-positive projection neurons that give rise to the "direct pathway" of the basal ganglia (mostly matrix neurons) and to the pathway from striosomes to the region of the substantia nigra, pars compacta (Berretta et al., 1992; Cenci et al., 1992; Johansson et al., 1994). Almost no

striatal interneurons show such an induction. To determine whether there was a change in this phenotypic profile following chronic cocaine exposure, we carried out double staining for these proteins and major phenotypic markers of striatal projection neurons and interneurons. We chose the timepoint of 1 week withdrawal following 7 day chronic cocaine treatment for quantitative comparisons with acutely treated controls, but also sampled other timepoints.

We found no major change in the phenotype of projection neurons expressing c-Fos-, JunB-, and FRA-like immunoreactivities. All were colocalized with dynorphin immunoreactivity (Figures 3D–3F). There were only small numbers of doubly labeled enkephalinergic projection neurons, and these declined with chronic treatment.

By contrast, there were prominent changes in inducibility of Fos–Jun proteins in striatal interneurons expressing nitric oxide synthase (NOS). The numbers of NOS-positive neurons expressing c-Fos-like and JunB-like immunoreactivities roughly doubled (241% for c-Fos, 189% for JunB, relative to numbers in acutely treated rats). With FRA immunostaining (Figure 3G), we found a 407% increase in NOS-positive neurons exhibiting intense FRA-like immunoreactivity. Increases (174%) were also found for persistently expressed FRAs in rats treated chronically and then maintained for 1 week of withdrawal without further challenge. There were no changes for the other classes of interneuron tested.

These results suggest that the network-level shift in patterns of inducibility of Fos–Jun proteins following chronic cocaine treatment are accompanied by shifts in expression in local circuit neurons within the striatum and that persistent FRAs follow the same shifts.

Extended Cocaine Exposure Induces a Continued Pattern Shift in Fos–Jun Family Proteins and Increased Expression of Dynorphin in Striosomes

To determine whether more extensive exposure to cocaine might fully down-regulate the inducibility of JunB and FRAs in the striatum, we exposed a group of rats to 2 weeks of the standard cocaine regimen (25 mg/kg, b.i.d.) and challenged the rats with a final dose of cocaine 18 hr later. Even with this doubling of the length of exposure, JunB-like and FRA-like immunoreactivites were still inducible, and the shift toward striosome predominance was still present. c-Fos-like immunoreactivity remained almost undetectable. These results indicate that the differential effects of chronic cocaine treatment on c-Fos-, JunB-, and FRA-like proteins were not peculiar to the 4 day or 1 week treatment protocols, but reflected a condition that holds even for longer periods of time.

Earlier work has shown that chronic treatment with cocaine increases the striatal expression of prodynorphin mRNA and dynorphin-like immunoreactivity (Sivam, 1989; Smiley et al., 1990; Steiner and Gerfen, 1995), particularly in striosomes (Daunais and McGinty, 1995). We found an obvious increase in striosomal expression of dynorphin-like immunoreactivity throughout most of the striatum in the rats treated for 2 weeks (Figures 3C and 3C′). Thus, changes in neuropeptide expression occurred in the same groups and phenotype of projection neuron as the changes in Fos–Jun family proteins.

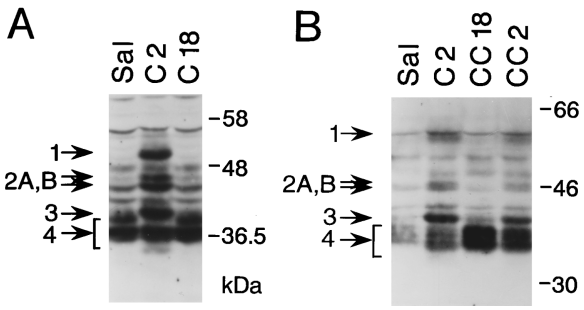

Figure 5. Western Immunoblots (FRA M Peptide Antiserum) of Caudoputamen Tissue Extracts Obtained from Saline-Treated Control Rats (Sal) or Cocaine-Treated Rats (C)

Cocaine (25 mg/kg intraperitoneal) was administered as acute treatment (C2 or C18) or chronic (CC2 or CC18) treatments. Striatal extracts were prepared either 2 or 18 hr after the last dose of cocaine was given. All experiments were repeated at least four times. Arrows indicate 55 kDa (1), 45 kDa (2), and 40 kDa (3) bands, as well as a 32–37 kDa cluster of bands (4).

Western Blotting Analysis of the Effects of Acute and Chronic Treatment on the Expression of Fos–FRA Proteins in the Striatum

To make a direct comparison between immunohistochemical and immunoblotting results with the M peptide FRA antiserum, we carried out parallel immunoblotting experiments with whole-cell protein extracts from the striatum of rats treated acutely or chronically with cocaine or saline.

Three series of bands were induced by acute cocaine treatment at 2 hr survival times, the largest of which (~55 kDa) may represent c-Fos (Figures 5A and 5B, band 1). This band had completely disappeared by 18 hr (Figure 5A). A second cluster of bands, at 40–45 kDa, showed elevated intensity 2 hr after acute cocaine (Figures 5A and 5B, bands 2 and 3), but had significantly decreased by 18 hr (Figure 5A, bands 2 and 3). The 40 kDa protein remained elevated above control levels at 18 hr (Figure 5A, band 3). A third cluster of cocaine-induced bands was detected migrating in the 32–37 kDa range (Figures 5A and 5B, band 4). Following 1 week of chronic cocaine treatment, these bands were also visible 2 hr after a cocaine challenge, but the 55 and 45 kDa bands were weaker than those observed in acutely treated rats (Figure 5B). By contrast, there was strong induction of the 40 and 32–37 kDa bands (Figure 5B), which remained elevated relative to controls at 18 hr (Figures 5A and 5B).

Discussion

Our findings suggest that at least three types of alteration occur in the striatum as a result of chronic exposure to cocaine: changes in the levels of expression of inducible transcription factors available for activating protein 1 (AP-1) binding within striatal neurons, changes in the inducibility of these transcription factors by a

subsequent challenge with cocaine, and changes in the neural circuits that express these proteins on cocaine challenge. Remarkably, although many of the quantitative changes reverted toward control levels during the withdrawal period following drug treatment, the network-level changes, like the behavioral sensitization induced by chronic treatment, did not. We suggest that these network-level changes reflect long-term modifications in the functional reactivity of basal ganglia circuits and that the changes may contribute to the expression of behavioral sensitization.

A Dynamic Transcription Factor Dimerization Code May Be Induced by Chronic Cocaine Treatment

An important feature of our immunohistochemical analysis was that we could identify the phenotypes of striatal neurons expressing the different protein immunoreactivities. The phenotypic profile suggested that the expression of the three protein immunoreactivities largely occurred in overlapping populations of neurons. It is thus highly likely that psychomotor stimulant exposure results in changes in expression of the different AP-1 proteins within single neurons of these classes. This is a critical point, because it suggests that changes in dimerization patterns of these proteins can occur in response to the chronic treatment. Such different heterodimeric complexes could, in turn, recognize different variants of AP-1 and CRE regulatory sites located in the promoter regions of target genes and lead to changes in their transcriptional regulation.

Our findings further indicate that there is not one change in AP-1-binding proteins available for potential dimerization but rather, a complex and continually evolving group of changes in these proteins and their expression patterns, both during drug treatment and during withdrawal. This temporal variation suggests that different networks of target genes may be hierarchically activated and/or repressed during chronic cocaine treatment and withdrawal in a time-dependent manner. Such time-varying combinations of regulatory events could ultimately be responsible for progressive and long-lasting changes in neuronal organization induced by the drug treatment. Interestingly, however, none of the changes in level of expression followed the known time course of behavioral sensitization, which begins soon after initiation of treatment and persists through withdrawal periods even longer than the 2 week period we chose (Kalivas and Duffy, 1993).

In agreement with Hope et al. (1994), Rosen et al. (1994), and Nye et al. (1995), we found that chronic cocaine treatment induced major alterations in the composition of the protein bands detected with the FRA antiserum, some of which may correspond to previously identified "chronic FRAs" (Hope et al., 1994; Nye et al., 1995). However, we were consistently able to detect the induction of 32–37 kDa FRAs even after acute cocaine treatment and could not find convincing evidence for the existence of 32–37 kDa FRAs specifically induced by chronic, but not by acute, cocaine treatment (Hope et al., 1994; Nye et al., 1995). The bands we detected in the 40–50 kDa range, and especially the 40 kDa band, may constitute a different group of persistently expressed FRAs.

Chronic Cocaine Treatment Induces Network-Level Changes in Inducibility and Expression of bZIP Proteins in the Striatum

Although our Western immunoblots and quantitative immunohistochemical analyses showed net increases or decreases in levels of expression of Fos–Jun family proteins, the distribution analysis suggests that both local increases and local decreases occurred for each protein class depending on the ensemble of striatal neurons examined. There was a clear down-regulation of inducibility in parts of the matrix compartment and, in the same brains, an up-regulation of inducibility in parts of the striosomal compartment. Thus, our findings suggest that a consistent, long-lasting change in the compartmental expression patterns of induced and persistent striatal Fos–Jun proteins is a key feature of the striatal response to chronic cocaine treatment.

The change in distribution pattern was already detectable after 4 days of cocaine treatment, and it persisted after cessation of treatment for the full 2 week withdrawal period we analyzed. This time course closely matches that of behavioral sensitization observed in a similar protocol by Kalivas and Duffy (1993) and noted qualitatively by ourselves. The distribution change appeared to be a property of the compartment phenotype per se. The change was qualitatively similar for all the protein immunoreactivities we studied, despite the fact that individual protein species differed sharply from each other in the quantitative aspects of their regulation. Even the severely down-regulated c-Fos-like immunoreactivity, when it became inducible again during withdrawal, showed the ensemble shift toward striosome predominance. The pattern change was evident also for FRAs expressed persistently in the absence of further challenge.

These ensemble-level alterations were associated with two clear changes at the single-neuron level. First, dynorphin-positive projection neurons were affected, some being recruited to express these proteins (neurons of some striosomes), others (neurons in parts of the matrix) becoming refractory to the induction of detectable levels of the Fos–Jun proteins. Second, there was a marked recruitment of NOS-containing interneurons. This result is particularly interesting because these neurons are local network inhibitory interneurons that have matrix-predominant distributions, tend to lie at striosome–matrix borders, and are thought to generate feedforward inhibition to the matrix compartment (Kawaguchi et al., 1995). Thus, they potentially could generate part or all of the down-regulation in the matrix, which contributed to the general ensemble shift in expression that we found.

Altered Dopamine Receptor Signaling and Receptor Interactions May Be Involved in the Regulatory Changes in bZIP Protein Expression

The molecular mechanisms by which chronic cocaine treatment recruits striosomal neurons and removes matrix neurons from the pool of responsive striatal neurons

are unknown. One obvious possibility is that, following chronic cocaine treatment, the relative efficacy of the D1-class dopamine receptor pathway is augmented in striosomes but is reduced in the matrix. An interesting alternative possibility is that the ensemble-level change reflects an altered balance of D1-class and D2-class dopamine receptor function in the striatum. The one other situation in which a noncompartmental pattern of striatal immediate-early gene induction has been experimentally converted into a patchy, striosome-predominant pattern is when D1-class and D2-class agonist treatments have been combined (Paul et al., 1992; LaHoste et al., 1993; Wirtshafter and Asin, 1994; see also Zhang et al., 1992; Dilts et al., 1993). The pattern evoked by combined D1–D2-class dopamine receptor agonists in normal rats is similar to that seen in rats treated with acute amphetamine and, as we show here, to that seen when the patterns induced by acute cocaine become transformed to a striosome-enhanced pattern with chronic cocaine treatment. Interestingly, rats given D1- and D2-class agonists in combination show enhanced behavioral stereotypy, as do rats sensitized by chronic exposure to cocaine. Such a shift in D1–D2 balance could be precipitated by different relative changes in dopamine release and/or reuptake (Robinson and Becker, 1986; Kalivas and Stewart, 1991).

The Changes in Transcription Factor Expression Induced in the Striatum by Cocaine Could Reflect Behaviorally Important Functional Changes in Basal Ganglia Circuits

Although our findings only show a correlation between the pattern change in striatal gene expression and behavioral sensitization, they do raise the possibility that the pattern change reflects neuroplasticity in the basal ganglia contributing to the behavioral change. A striking feature of the network-level change we found is that it so closely parallels the time course of behavioral sensitization. To date, no other single neurological marker of pre- or postsynaptic function in the striatum has been found to parallel so fully the build-up and maintenance of sensitization during withdrawal from cocaine (see Robinson and Becker, 1986; Kalivas and Stewart, 1991; Kalivas and Duffy, 1993). In our experiments, we found this as well for the changes in inducibility of Fos–Jun proteins and for the persistent expression of FRAs. The inducibility and the expression of these proteins were dramatically altered, but the alterations lacked the combination of early onset and prolonged maintenance shown by behavioral sensitization. However, when we used the gene induction assay anatomically, as a way to identify which neurons were activated to express the genes on challenge, we found that a network-level shift toward striosomal activation that did have the temporal characteristics of the behavioral change.

Behaviorists have suggested that the increased stereotyped behavior exhibited by animals that become sensitized to psychomotor stimulants depends on the caudoputamen (Robinson and Becker, 1986; Kalivas and Stewart, 1991). Our findings suggest that, within the caudoputamen, an increased relative responsiveness of striosomal neurons to cocaine challenge emerges in the sensitized animal. This correlation is interesting from the behavioral point of view, because the known properties of the striosomal compartment of the striatum make it plausible that augmented activation of striosomal functions might play a part in the stereotyped behaviors typical of the sensitized animal. Relative to the matrix of the caudoputamen, striosomes have preferred connectivity with key elements of the limbic forebrain, including cortical regions that, in the human, have been implicated in the altered metabolic activity associated with abnormal repetitive behavioral patterns (reviewed by Eblen and Graybiel, 1995).

Experimental Procedures

Adult male Sprague–Dawley rats (Charles River Labs, Wilmington, MA) initially weighing 180–200 g were housed in pairs at the MIT animal facility under a 12 hr light/dark cycle with free access to food and water.

Rats were treated with intraperitoneal injections of cocaine HCl (25 mg/kg, Sigma, St. Louis, MO) twice a day for 4, 7, or 14 days. The rats received either no challenge or a challenge dose of cocaine 18 hr after the last administration and were euthanized 2 or 18 hr later. To analyze the effects of drug withdrawal, rats were treated for 7 days with cocaine as described above and were given withdrawal periods of 18 hr or 3, 7, or 14 days with a final cocaine challenge before euthanasia 2 or 18 hr later. Some rats received an intraperitoneal injection of saline or no treatment in place of the cocaine challenge. Control rats were treated with two daily intraperitoneal injections of saline and a final cocaine or saline challenge. Other rats were treated acutely with cocaine (25 mg/kg) or with D-amphetamine sulfate (5 mg/kg, Sigma). The doses of 25 mg/kg cocaine and 5 mg/kg amphetamine were chosen on the basis of previous experiments with acute treatment (Graybiel et al., 1990; Moratalla et al., 1992, 1993). The behavioral reactions of the animals were noted during the survival time.

Immunocytochemistry

Brains were fixed by transcardial perfusion with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), cryoprotected, and cut transversely at 20 μm on a sliding microtome. In all, the brains of 190 rats were analyzed.

Single antigen immunostaining was performed on free-floating sections as described earlier (Graybiel et al., 1990), with a standard avidin–biotin peroxidase (ABC) protocol (Vectastain, Vector Laboratories, Burlingame, CA). The peroxidase was detected with diaminobenzidine (DAB, Sigma) with nickel intensification. Two-color dual antigen immunostaining was obtained by serial ABC staining reactions with final steps in nickel-intensified DAB (purplish gray) and in DAB alone (brown). For NOS-containing interneurons, sections were first immunostained by the ABC protocol and then reacted for NADPH diaphorase activity histochemically (Xu et al., 1994).

Antisera

To localize Fos–Jun family proteins, we used polyclonal rabbit antisera against c-Fos (1:200, Oncogene Science, Manhasset, NY), JunB (1:4,000, provided by Dr. R. Bravo, Bristol-Myers, Princeton, NJ), and the M peptide sequence shared by members of the Fos family (1:5,000, provided by Dr. M. I. Iadarola, National Institutes of Health, Bethesda, MD). To localize striatal projection neurons, we used antisera against dynorphin B 1–29 (1:10,000, provided by Dr. S. Watson, University of Michigan, Ann Arbor, MI), Met-enkephalin (1:2,000, INCSTAR, Stillwater, MN), and calbindin D_{28K} (1:3,000, provided by Dr. P. Emson, Medical Research Council, Brabaham, United Kingdom). To identify striatal interneurons, we used monoclonal mouse antiserum against parvalbumin (1:1,000, Sigma) and polyclonal goat antiserum against choline acetyltransferase (ChAT, 1:100, Chemicon, Temecula, CA).

Western Immunoblotting

Striata were rapidly dissected from brains removed after decapitation and sonicated in 10 vol of buffer (Hope et al., 1994). After centrifugation, supernatant proteins were resolved by SDS–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (MSI, Westboro, MA). Membranes were incubated with anti-FRA primary antiserum (1:4,000) (Young et al., 1991) and subsequently with HRP-conjugated goat anti-rabbit secondary antibody (Bio-Rad, Richmond, CA, 1:4,000). Immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

Image Analysis and Cell Counting

Counting of immunopositive nuclei was done single-blind with a 10× objective with a Biocom imaging system (Les Ulis, France) at a standard transverse level, ~10 mm rostral to the interaural line. A horizontal strip through the midheight of the caudoputamen was chosen as the sample area for all sections. Before counting, the images were thresholded at a standardized gray-scale level empirically determined by independent observers to allow detection of nuclei stained with moderate to high intensity, with suppression of lightly stained nuclei. The unprocessed color video image and the microscope image were also viewed directly to resolve ambiguities.

Acknowledgments

This work was funded by National Institute on Drug Abuse grant DA08037 and the Tourette Syndrome Association. We are grateful to Mr. Glenn Holm for the cell counting; to Mr. Henry Hall, who is responsible for the photography; to Miss Diane Major for her help with the immunohistochemistry; and to Mr. Andrew Tan for his help with the experiments. We thank Drs. M. Iadarola, R. Bravo, S. Watson, and P. Emson for their generous gifts of antisera.

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Received January 31, 1996; revised May 10, 1996.

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